ON THE OCCURRENCE OF TWO SPECIES OF PARASITES IN EQUINE "PIROPLASMOSIS" OR "BILIARY FEVER."

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(With Plate III, 8 Diagrams, 1 Text-figure and 5 Charts.)

"Piroplasmosis," or "biliary fever" in horses, has hitherto been regarded as a disease due to a distinctive parasite, *Piroplasma equi* Laveran. We propose to show that there are two specifically distinct parasites concerned in the production of biliary fever, and that consequently two distinct diseases have hitherto been confused under this name and under the name of piroplasmosis.

The parasite described as *Piroplasma equi* by Laveran (1901, p. 385) was studied by him in stained blood-films sent to him from South Africa by Theiler. Laveran referred the parasite to the genus *Piroplasma,* which, even at present, is made to include parasites which are distinctly different from this genus. As Nuttall and Graham-Smith have shown, parasites belonging to the genus *Piroplasma,* of which *P. bovis* is the type species, invariably multiply in a characteristic manner in that the peculiar piriform parasites develop through a process resembling budding from a large, rounded, or slightly amoeboid parasite the whole of whose protoplasm flows into the "buds" and gives rise as a rule to two piriform parasites without a residual body being left. As species of true *Piroplasma* we have hitherto recognised *P. bovis*, *P. canis* and *P. pitheci*[1]. These species occur inside the corpuscles grouped in large numbers in pairs of piriform parasites connected at their pointed extremities. In *P. canis* a considerable number of corpuscles contain two pairs of piriform parasites, and at times higher multiples may be encountered.

Laveran's description and figures show that his *Piroplasma equi* does not possess the characteristic mode of multiplication and form of a true *Piroplasma*. The mode of multiplication as described by Laveran from stained specimens alone is moreover inaccurate. Convinced of the difference between Laveran's parasite and *Piroplasma*, França (1909) has more recently placed the parasite in a new genus, named *Nuttallia*. The parasites belonging to this genus do not multiply according to the method described for *Piroplasma*; they do not occur as pairs or multiples of pairs of piriform parasites inside of corpuscles, and they form distinctive "*cross forms*" which Bowhill (1905) and França (1909) regard as multiplication forms. According to França, then, *Piroplasma equi* Laveran 1901 = *Nuttallia equi* (Laveran) França 1909.

That two types of parasites occur in equine piroplasmosis was pointed out by Robert Koch (1905, p. 1867) who believed that they might cause distinct diseases, and here the matter rested until December 1910 when we published a short preliminary note upon the results of our investigations, which clearly show that two distinct species of parasites occur in horses suffering from biliary fever, and consequently that two distinct diseases are included under this name.

Through the courtesy of Sir John McFadyean, of the Royal Veterinary College, London, we obtained a strain of *N. equi* derived from South Africa and maintained by passage through several horses in London. The strain was sent to Cambridge in citrated blood taken from a horse which had been inoculated with a positive result two years before.

A detailed study of this parasite, both in fresh blood preparations and in stained films, quickly convinced us that França's view is correct, namely, that *P. equi* Laveran is not a *Piroplasma*, and we of necessity concur in referring the parasite to the genus he has done one of us the honour of naming *Nuttallia*. Our studies upon the living parasite, moreover, confirm the view that the cross forms are multiplication forms as the sequel will show.

So as to avoid confusion we shall first of all describe our observations on *N. equi* (Laveran 1901) and afterwards deal with the true

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[1] Nuttall and Graham-Smith (vi. 1908), p. 134 etc.

Piroplasma (*P. caballi* Nuttall 1910) which also occurs in horses suffering from "biliary fever." As one of our *P. caballi*-infected horses was inoculated with *N. equi* after it had recovered from the clinical symptoms produced by the first-named parasite, it will make matters clearer to show the course of the experiments in the form appended:—

Nuttallia equi

Horse I. Inoculated 27. xi. 1909 with blood obtained from Sir John McFadyean. Parasites appeared 8th day. Horse died 11th day.

Horse II. Inoculated 8. xii. 1909 with blood from *N. equi* Horse I. Parasites appeared 7th and disappeared 22nd day. Horse recovered, and is still alive, 27 February, 1912.

Horse III. Inoculated 2. viii. 1910 with← blood from *N. equi* Horse II. Parasites appeared 10th day. Horse died 20th day.

Piroplasma caballi

Horse I. Inoculated 4. vi. 1910 with blood obtained from Dr E. J. Marzinowsky. Parasites appeared 15th day. Horse died 19th day: 22. vi. 1910.

—*Horse II.* Inoculated 22. vi. 1910 from *P. caballi* Horse I. Parasites appeared 8th day. Horse recovered. (Subsequently inoculated with *N. equi*[1].)

Horse III. Inoculated 18. vii. 1910. Parasites appeared 9th day. Horse died 19th day.

[1] *N. equi* Horse III is the same animal as *P. caballi* Horse II. The *P. caballi* strain was carried on from Horse II to III before *P. caballi* Horse II was inoculated with *N. equi*.

I. NUTTALLIA EQUI (Laveran).

(A) *The living parasites.*

Although we spent roundly 200 working hours in the continuous study of the living parasite in fresh blood-films maintained at body-temperature, the results of our observations can be summarized briefly. The following summary is based on the continuous observation, coupled with graphic records[1], carried out on

	Average duration of observation on each parasite in minutes
41 small and medium-sized parasites	79
43 medium and large-sized parasites	106
8 pairs of small and medium-sized parasites	83
38 dividing and cross-forms	95

The number of infected corpuscles as compared to uninfected ones was small. Thus, in Horse I the number of infected corpuscles did not exceed 5·4 %; in Horse III they attained 13·2 %. In films obtained from another horse, and which were kindly lent us by Sir John McFadyean, there were 6·6 % of infected corpuscles. In the case of Horse II the parasites appeared in the blood in such small numbers that there was no adequate opportunity of studying them.

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[1] The accompanying diagrams are selected from a large series of graphic records.

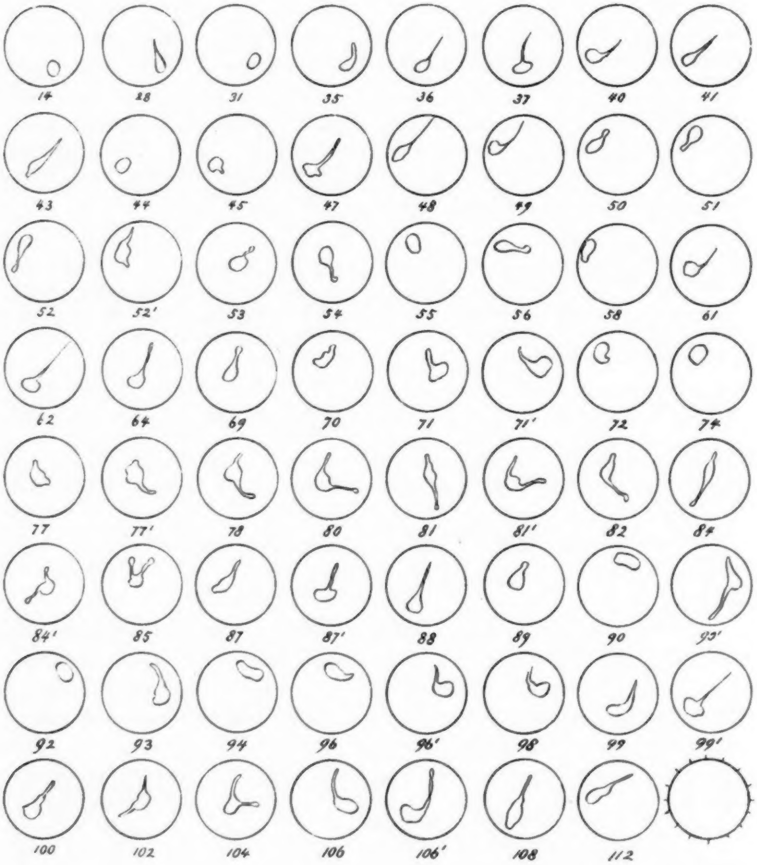

Diagram I. N. equi. Illustrating the movements and alterations of form of a small parasite which was under observation from 14 minutes until 112 minutes after the blood was drawn. The corpuscle finally became crenated. The parasite appeared alternately oval, piriform and amoeboid, etc. protruding at one time two pseudopodia. The parasite seemed to grow in size. (Horse I, 6. XII. 1909, G. H. F. N. del.)

(1) *Small parasites* (Diagram I) measuring about 1 to 1·4 μ in size are frequently observed to be amoeboid. They move about within the corpuscle in an irregular manner whilst altering their shape, being at different times either oval, rounded, or piriform; or they may protrude a slight bud-like process. Some of this alteration of form is attributable to the rotation of the parasites within the corpuscle. The parasite may occasionally protrude a long filiform process, more rarely do they protrude two bud-like or filiform processes. These processes may be protruded or retracted slowly or within a couple of minutes. In some cases a rounded parasite was seen to protrude a long straight or curved filiform process extending two-thirds across the width of a corpuscle, the parasite undergoing but very little alteration in form for upwards of three hours afterwards. The thread-like processes at times showed a terminal swelling. When the parasites assumed a piriform shape it was only transitorily. The escape of small single parasites from corpuscles was never witnessed, and small parasites were never seen to divide. In some cases we believe that the parasites increased slightly in size during the time they were under observation.

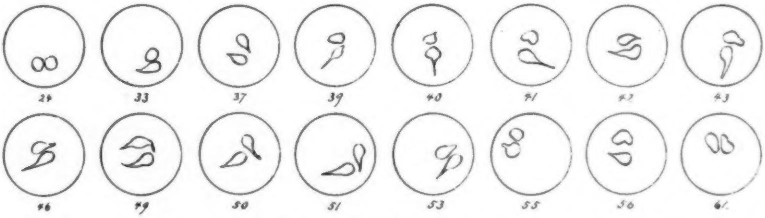

Diagram II. N. equi. Illustrating the behaviour of two small parasites within a corpuscle during the period 24 to 61 minutes after the blood was drawn. The parasites appeared alternately rounded, piriform and of irregular form. (G. H. F. N. del.)

When two small or medium-sized parasites occur within a corpuscle (Diagram II) they behave in a manner similar to that of the single parasites above described.

(2) *Single medium-sized and large parasites* (Diagrams III–IV) behave very much as do the small ones. They change their shape more or less rapidly from round to oval or piriform, or they protrude one or two long processes which may or may not possess terminal swellings. We rarely observed the protrusion of three pseudopodia by amoeboid parasites.

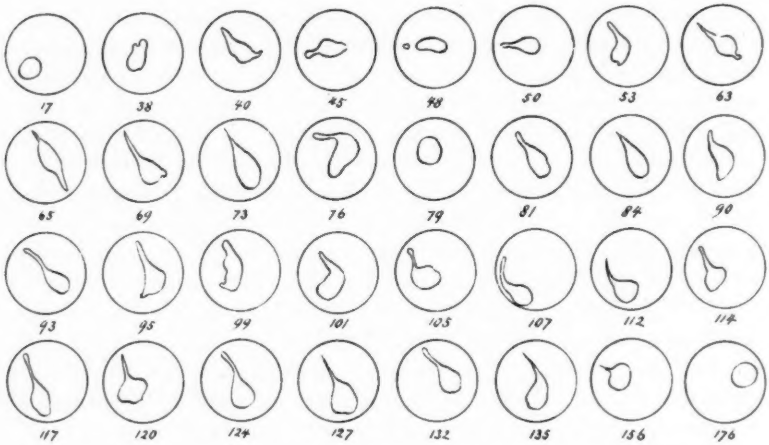

Diagram III. *N. equi*. Illustrating the behaviour of a large parasite within a corpuscle during the period 17 to 176 minutes after the blood was drawn. The parasite appeared alternately rounded, amoeboid and piriform, and finally became rounded when all activity ceased. (Horse I, 6. XII. 1909. G. H. F. N. del.)

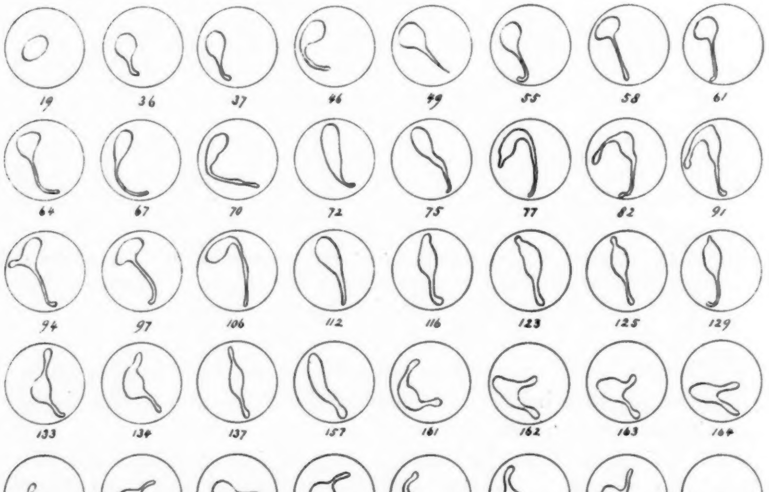

Diagram IV. *N. equi*. Illustrating the behaviour of a large parasite within a corpuscle during the period 19 to 237 minutes after the blood was drawn. The parasite appeared oval at first, then protruded a pseudopodium which caused it at times to appear piriform; then it protruded two pseudopodia so that it somewhat resembled a dividing form of *Piroplasma*. Finally, it became rounded and ceased to exhibit movements. (G. H. F. N. del.)

(3) *Very large single parasites,* of which a certain number are to be found, may show very little change of form, although they be watched for hours. They may remain rounded for long periods of time or assume an elongate[1] or plump piriform shape which is usually more or less transitory.

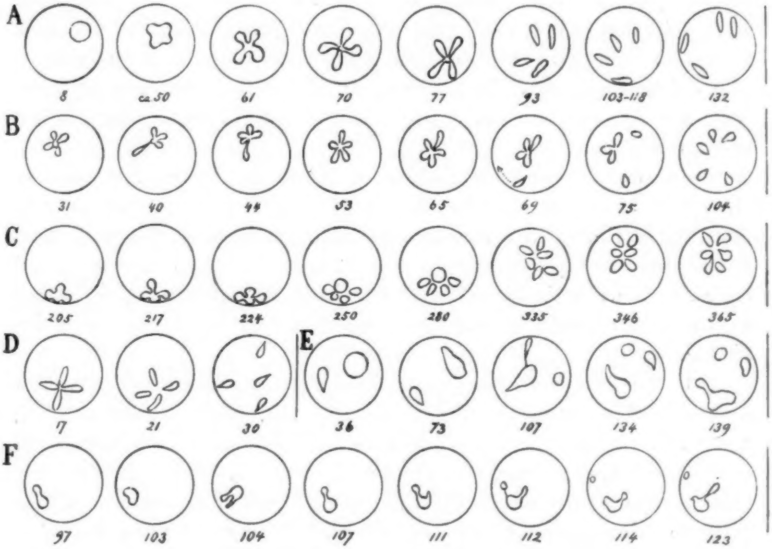

Diagram V. N. equi. Illustrating more especially the mode of multiplication of *N. equi* as observed in several infected corpuscles.

Fig. A. A single parasite observed through successive stages to the formation of four free oat-shaped or piriform parasites (Horse III, 13. VIII. 10, blood at 41° C., G. H. F. N. del.).

Fig. B. A "cross-form" which gradually gives rise to five parasites which move about inside the corpuscle (Horse I, 7. XII. 09, 39° C., H. B. F. del.).

Fig. C. The later stages in the development of a single parasite into six parasites (Horse I, 6. XII. 09, 38° C., C. S. del.).

Fig. D. A "cross-form," of which the component elements separate and swim about actively in the corpuscle prior to escaping without injuring the corpuscle (Horse III, 13. VIII. 10, 35° C., G. H. F. N. del.).

Fig. E. Whereas in the preceding instances the mother cell divides directly into 4–5–6 daughter cells of equal size, we have in this case what would appear to represent a budding-off process of small daughter cells which are liberated successively (Horse III, 12. VIII. 10, 37° C., G. H. F. N. del.). In Fig. F apparently a similar process is taking place (Horse II, 17. XII. 09, 37° C., H. B. F. del.).

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[1] Such parasites may extend across the whole width of the corpuscles. The latter, in the horse, average $5{\cdot}6\mu$ in diameter.

(4) *Dividing forms*: In a number of cases stages of development of a single parasite into four were observed, and the four young parasites were seen to separate. In one case (Diagram V, A) a previously rounded parasite was seen to give rise to four parasites after developing into a "cross-form." *In most cases* the cross-forms break up into four distinct parasites which wander away from each other. In three cases, what appeared to be an ordinary cross-form, gave rise to five parasites (Diagram V, B), and in one case six parasites resulted from the process of division (Diagram V, C). Young parasites, soon after they separate, may be very active and move about within the corpuscle (Diagram V, D).

In a few cases multiplication occurred by a budding process (Diagram V, E and F). Appearances may well be deceptive in such cases, for at times, when the infected corpuscle ruptures, the main mass of the parasite's protoplasm is seen to be connected to the supposed daughter individuals by delicate strands of protoplasm, which, owing to the haemoglobin within the corpuscle, had previously been invisible.

(5) *The escape of young parasites from corpuscles* was observed in a number of instances to occur shortly after they had become separated, the young parasites having arisen from a cross-form. The parasites did not rupture the corpuscle in the act of escaping. Immediately after their escape, singly or all together, the parasites appeared ovoid or piriform; they at times swam about actively for a few moments, after which they usually vanished, the conditions *in vitro* being doubtless unfavourable to their continued existence. Although we are convinced that the young parasites under natural conditions immediately re-enter fresh corpuscles after the manner of *Piroplasma*, we did not actually succeed in observing their entry into another corpuscle. The minute size of the parasites left us in doubt as to whether they lay upon or within the fresh corpuscle. On one occasion we saw a medium-sized parasite escape and degenerate outside the corpuscle which it left uninjured. It was noted several times in observing the microscopic field that the first corpuscles whose contained parasites escaped were those that harboured four young parasites. Some of these corpuscles vanished whilst the contained parasites appeared to be motionless, in other cases the corpuscles burst suddenly and the parasites were all ejected into the plasma.

(B) *The stained parasites.*

Having studied the living parasite, we were in a better position than previous observers to understand the significance of the forms which are encountered in stained films. Certain forms, however, still require investigation. Reference to the protocols relating to the horses upon which we experimented (see Appendix, *N. equi* Horses I–III, pp. 85–89 and charts) shows the results of the examination of their blood during the course of the disease induced by this parasite.

The parasites stain in a similar manner to *Piroplasma.* The hyaline cytoplasm takes on a blue colour with Giemsa's stain and the nuclear structures are stained deep carmine. The blue staining cytoplasm may appear more or less vacuolated or condensed peripherally. Definite nuclear changes precede the division of the cytoplasm; these changes are considered on p. 75 (Diagram VII) and p. 84 in the description of Plate III.

Time when the parasites appear in the blood. Percentage of infected corpuscles.

Horse I. The parasites appeared in the blood on the morning of the eighth day after inoculation, roundly 0·6 °/₀ of the corpuscles being found infected in the afternoon. They gradually increased in numbers up to the afternoon of the tenth day, when 5·4 °/₀ of the corpuscles were infected; and on the eleventh day, when the horse died, they had decreased slightly in numbers.

Horse II. The parasites appeared on the seventh day after inoculation. They gradually increased in numbers so that roundly 1 °/₀ of the corpuscles were found infected on the tenth and eleventh days. Their number then fell rapidly so that only isolated parasites could be detected up to and including the 21st day, after which they disappeared and the horse recovered.

Horse III. The parasites appeared on the tenth day after inoculation, 0·2 °/₀ of the corpuscles being found infected. They increased in numbers up to the twelfth day, when 11·6 °/₀ of the corpuscles contained them. They persisted in fair but somewhat variable numbers (5 to 13 °/₀ of the corpuscles being infected) from the 13th to the 20th day, when the horse died.

The number of infected corpuscles enumerated in these horses attained at most 5·4 °/₀, 0·9 °/₀ and 13·2 °/₀, respectively. The parasites in Horses I, II and III appeared with the onset of fever; their number rose and fell with the body-temperature as will be seen by reference to the charts given in the Appendix.

The types of parasites encountered in the blood.

Owing to the changes of form observed by us in living parasites we have not attached much importance to the different shapes seen in parasites which have been fixed and stained. Naturally, they may assume a great variety of forms in death, these forms corresponding to what has been described in the living parasite. Enumerations of parasites according to their relative size lead us to no definite conclusions with regard to the relative prevalence of small, medium or large parasites at any stage of the disease. In Horse I the medium and large-sized parasites predominated over the small until the day of death, when there was an increase in small parasites; in Horse II the large parasites predominated more or less considerably over the small forms in 15 out of 19 counts, and there was no increase of small forms toward death[1]. About 90 °/₀ of the infected corpuscles contained single parasites of all shapes and sizes, usually 2–5 °/₀ contained 2 to 4 parasites and 1–5 °/₀ contained dividing or cross-forms[2]. Whereas in Horse III only few free parasites were usually encountered (0·2 to 6 °/₀), in Horse I as many as 26 °/₀ of free parasites were encountered on the day of death, thus indicating that the corpuscles were rapidly breaking up and liberating the contained parasites[1].

These enumerations of the different types of parasites encountered in the blood offer a marked contrast to what is observable in *Piroplasma* (see p. 81).

The accompanying Diagram VI represents what appears to be the usual mode of multiplication of *N. equi* in the circulating blood, judging from the observations made in the first instance upon living parasites, and in the second upon stained specimens. The reader is referred to Plate III and its accompanying description for further details.

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[1] In this connection it should be noted that Horse III had haemoglobinuria and jaundice but died without marked lesions, whereas the contrary was the case in Horse I. The latter's spleen weighed 7½ lbs., the liver 12¼ lbs., and extensive abdominal haemorrhage was detected at autopsy.

[2] In films from South Africa and Italy kindly lent us by Sir John McFadyean and by Professor A. Negri (Pavia) respectively, the dividing or cross-forms represented 7·2 °/₀ and 4·4 °/₀ of the *N. equi* present in blood-films.

N. equi multiplies slowly and in the following manner:

(1) The minute piriform or oat-shaped parasite enters a fresh corpuscle and (Diagram VI, 2, 3, 4, 5) grows in size, being slightly amoeboid, with a general tendency to resume a pear-shape. Definitely amoeboid movements (6) are, however, only to be seen distinctly when the parasite has attained a certain size. Judging from the form of the chromatin masses, stages 7, 8, 9, 10 follow next. The rest of the cycle has been continuously observed in the living parasite: the formation and breaking-up of the cross-form, the scattering of the daughter cells within the corpuscle, and their escape from the corpuscle,

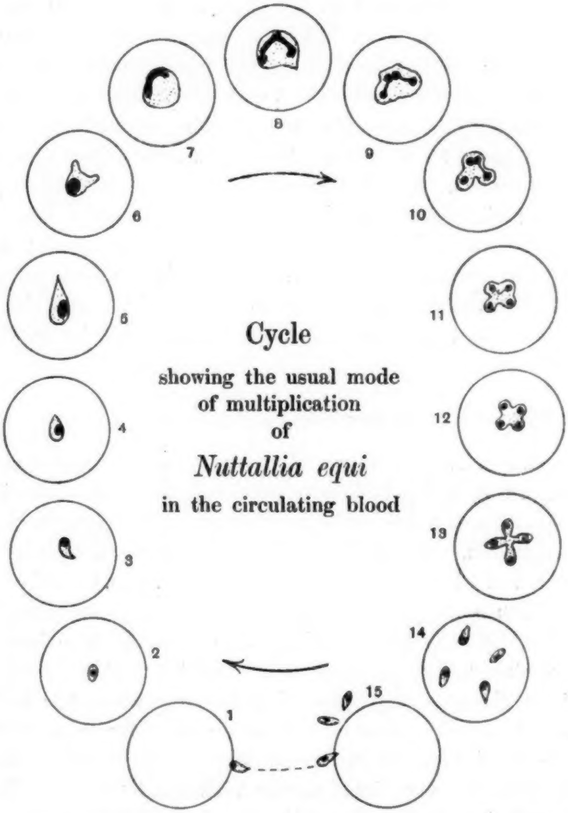

Diagram VI. Illustrating the usual cycle of development of *N. equi* in the circulating blood. (Consult text pp. 74–76.)

which usually does not appear to be injured in the process. As previously stated, we find that corpuscles containing four parasites are liable to "vanish" earlier than do others.

(C) *Distribution of the parasites in the body.*

Immediately after the death of the horses (Nos. I and III) smears were prepared from the internal organs and stained, and the percentage of infected corpuscles and of different types of parasites enumerated. The counts, which we need not give here, revealed no more than did those made from the peripheral blood during life. The percentage of infected corpuscles appeared to be fairly uniform in all the organs examined, viz. spleen, liver, kidney, suprarenal glands, lung, heart-muscle, brain, and blood taken from the jugular vein and heart, etc., gave similar counts to those obtained with peripheral blood. When the parasites were classified according to types, the predominant forms were small, ranging from 50 to 80 % of the total number of parasites observed in the organ smears. Judging from the percentage of extracorpuscular forms, the breaking down of the infected corpuscles in Horse I took place sooner than in Horse III on the approach of death; the blood from the various organs of Horse III contained from 14 to 22 % of the parasites free in the plasma, whereas at no time were more than 5 % of free parasites detected in the peripheral blood of this horse whilst the animal was alive.

Observations on the blood.

In the case of *N. equi* Horse III (refer to protocol and chart in the Appendix, p. 88), which had been previously infected with *P. caballi*, blood counts showed a fall in the number of red blood corpuscles as the disease progressed. When the parasites appeared there were roundly nine million red blood corpuscles present per c. mm.; on the 12th day they had fallen roundly to seven million, and the fall continued so that on the 19th day only three million were present. The haemoglobin showed an almost corresponding fall. The leucocytes were markedly increased on the two days preceding the day when the horse died. Differential leucocyte counts were made on only four days; they showed a marked decrease in the number of eosinophiles. Nucleated red blood corpuscles were found on the 17th and 19th day. The blood was not studied in Horses I and II. In Horse III there were haemoglobinuria and jaundice.

II. PIROPLASMA (BABESIA) CABALLI.

(A) *The living parasites.*

Although the scarcity of parasites in the blood of the infected horses prevented our making as complete a study of their development *in vivo* as we should have wished, we nevertheless convinced ourselves that *P. caballi* (Diagram VII) develops in a manner similar to *P. bovis* and *P. canis*. Small and medium-sized parasites are usually oval or rounded in shape; they may, however, assume a somewhat piriform shape momentarily. When such parasites escape from corpuscles they degenerate and die. When large single piriform parasites occur in corpuscles (Diagram VII, D) they move about slowly in the corpuscle whilst retaining their piriform shape; as in *P. bovis*, some large piriforms may extend almost across the whole width of the corpuscle. When moving about, such piriforms may occasionally appear rounded owing to their movements within the corpuscle being directed towards or away from the observer. Piriform parasites occurring in pairs were seen, as in *P. canis*, to escape from a corpuscle which afterwards vanished. Typical multiplication forms (Diagram VII, A and B) were seen as in *P. canis* and *P. bovis*, the "buds" persisting and leading almost to the complete formation of paired piriforms. The process of

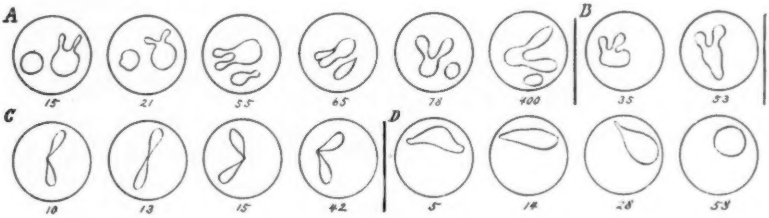

Diagram VII. P. caballi. Illustrating living parasites observed in fresh blood-films at blood temperature. (Horse I, VI. 1910.)

Fig. A. Two parasites, the larger, observed 15 minutes after the blood was drawn, almost developed into two piriform parasites at the end of 400 minutes. The smaller parasite underwent changes of form from round to piriform and oval. (G. H. F. N. del.)

Fig. B. A single parasite: early stages of multiplication observed 35–53 minutes after the blood was drawn. (C. S. del.)

Fig. C. Two conjoined piriform parasites showing changes of position within the corpuscle during 10–42 minutes. (G. H. F. N. del.)

Fig. D. Large piriform parasite slowly altering its shape and finally becoming rounded. The observation lasted 58 minutes. (C. S. del.)

multiplication, like that of *P. bovis,* is slower than in *P. canis,* but otherwise it is similar, and consequently requires no further description. Very large transitorily piriform parasites are frequently observed as in *P. bovis.*

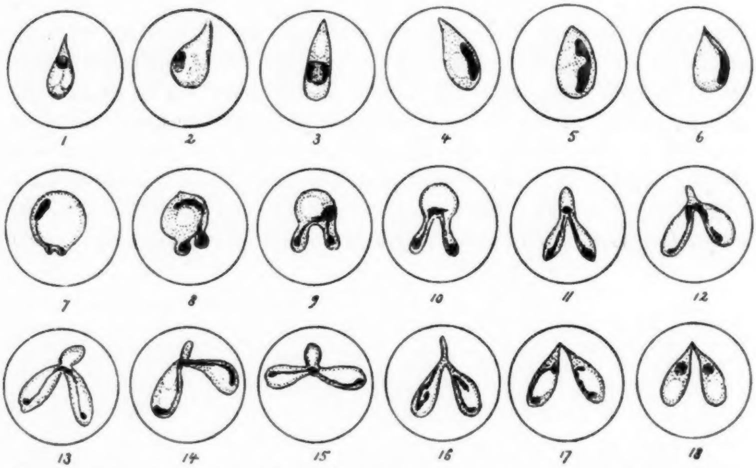

Diagram VIII. P. caballi. Illustrating appearances seen in *Giemsa-stained parasites.* Selected figures from a large series of coloured drawings by C. S. made from films derived from Horses I and III and from films supplied by Dr Marzinowsky. The figures show the mode of multiplication. The blue-stained protoplasm is indicated by light stippling, the deeply-staining chromatin by black, and the pink or faintly-staining chromatin by close stippling. The contours of the corpuscles are schematized.

Fig. 1. Single piriform parasite which has recently entered a corpuscle. A deeply-staining chromatin mass lies to one side of the pink-staining body and upon or within a small spherical mass of faintly staining chromatin.

Figs. 2–4. Single parasites which have developed in size. Such parasites may be rounded but usually assume a piriform shape whilst slightly altering their form. The chromatin mass increased in size and frequently becomes elongated.

Fig. 5. Single parasite showing a large irregular faintly pink-staining chromatin mass.

Fig. 6. Parasite with dark-staining chromatin mass prolonged into a thread which terminates into a bead-like mass.

Figs. 7–9. The chromatin protruded in a fork-like manner into the buds of blue-staining protoplasm. (Figs. 7–18 as in *P. canis, P. bovis* and *P. pitheci.*)

Figs. 10–15. Showing more advanced stages of multiplication, a large mass of chromatin being seen at the point of bifurcation at the base of the buds.

Fig. 16. Showing the separation of the chromatin masses in the daughter cells which remain connected at their pointed ends.

Fig. 17. Newly-formed daughter cells still containing drawn-out masses of chromatin.

Fig. 18. Mature pair of piriform parasites, and which are ready to separate and escape from the corpuscle.

(B) *The stained parasites.*

A study of the stained parasites (Diagram VIII) shows that they are very closely allied to *P. bovis*, the large parasites being less actively amoeboid than in *P. canis*, and tending to assume a piriform shape. The chromatin structures are identical with those of *P. bovis*. The reader is referred to the Appendix for particulars relating to the course of the disease in the horses upon which we experimented (*P. caballi* Horses I, II and III).

Time when the parasites appeared in the blood. Percentage of infected corpuscles, etc.

Horse I. The parasites appeared in the blood on the 15th day after inoculation; they had increased slightly in numbers by the 18th day. The horse died on the 19th day from pulmonary haemorrhage. The disease (piroplasmosis), being but in the initial stage, the organs showed no distinct lesions.

Horse II. A few parasites appeared on the eighth day after inoculation and then disappeared, the animal recovering.

Horse III. The parasites appeared on the tenth day after inoculation; by the 13th day only 6 per 30,000 corpuscles were found infected, after which there was an intermittent increase of parasites; but even on the 19th day, when the horse died, only 0·5 % of the corpuscles were found to contain parasites.

At autopsy, this horse showed marked jaundice of the mucous membranes and subcutaneous tissues; the lungs were congested, the kidneys oedematous; the spleen was much enlarged, semi-fluid and very friable; it weighed 15½ lbs.; the liver was enlarged.

A rise in body-temperature preceded the appearance of the parasites in the blood of the three horses.

The types of parasites observed.

Consideration of the types of parasites encountered in the peripheral blood during life shows that the predominant forms are round (O) or double piriforms (PP). In Horses I and II only (O) and (PP) were found in the blood, but they were very scarce. Other typical forms were however encountered in the blood of Horse III whilst it lived, and in this case counts of the different types were rendered possible, although the number of infected corpuscles only attained 0·5 %.

When we average the results of the counts of types of parasites made during four days on one of our horses suffering from *P. caballi* infection, we find the following percentages of the different types present in the blood, free parasites being omitted:

(P)	(O)	(D)	(PP)[1]
6·5	60·8	4·8	27·6

The average of three counts on blood-films prepared by Dr E. J. Marzinowsky, in Moscow, gave corresponding figures:

(P) (O)	(D)	(PP)
65	5	30

These percentages indicate roughly the relative duration of the various stages in the cycle of development of the parasite from the single piriform parasite on to the formation of (PP); the stages which last longest are the (O) and the (PP), that is why they yield a higher percentage in the counts. The duration of the (PP) stage is usually shorter than the (P) + (O) stage; thus in *P. caballi* the average of seven counts yield 67 % (P) + (O) to 32 % (D) + (PP). The (P) stage is usually short for the parasite soon becomes rounded or amoeboid after entering a fresh corpuscle. The proportion of free parasites is usually small except when the blood corpuscles are breaking up rapidly at the end of the disease, because the free Ps almost immediately re-enter fresh red blood corpuscles. When (PP)s are classified according as the parasites are conjoined at their pointed ends or separate in the corpuscle, we find that for every 100 conjoined pairs there occur only about ten separated piriforms—in other words the parasites usually escape soon after they cease to be conjoined.

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[1]

The signs	indicate corpuscles containing
(P)	a single piriform parasite.
(O)	a single rounded parasite.
(D)	a dividing parasite.
(PP)	two piriform parasites.

(C) *Comparison of types of parasites encountered in different species of Piroplasma.*

A comparison of the different species of *Piroplasma* in respect to the predominant types which are encountered in the peripheral blood, only *P. pitheci* being omitted, shows that they all agree fairly well:

Species of Piroplasma	% of types encountered						Reference	No. of counts averaged (each representing 250 to 500 parasites)
	(P)	(O)	(D)	(PP)	(OO)	(P_4)[1]		
Caballi	6·5	60·8	4·8	27·6	—	—	N. & S.	4
Caballi	65		5	30	—	—	N. & S.	3
Bovis	55		11	34	—	—	Nuttall & Graham-Smith (1908, p. 139)	2
Bovis	54		6	40	—	—	Nuttall & Hadwen (1909, p. 247)	7
Bovis	55		6	39	—	—	Nuttall & Hadwen (1909, p. 244)	7
Canis	5·0	45·0	0·7	43	1	4	Nuttall (1910, p. 423)	7
Canis	0·6	54·0	3	41	0·6	0·7	Nuttall & Hadwen (1909, p. 171)	7

If we group the numbers in two columns according as the infected corpuscles contain a single parasite or two or more parasites we obtain the following:

	Corpuscles containing		
	Single parasites	Two or more parasites	No. of counts averaged
P. caballi	71	29	7
P. bovis	61	39	7
P. canis	54	42	14

In the above table the forms (P), (O) and (D) are reckoned as one parasite. The results of the enumerations in the case of *Nuttallia equi* are totally different, even when we include the dividing or cross-forms in the second column; thus we obtain:

	Corpuscles containing		
	Single parasites	Two or more parasites and (D)s	No. of counts averaged
N. equi	96	4	7
N. equi	95	5	19

These counts, therefore, indicate, apart from the differences in morphology, that *Piroplasma* and *Nuttallia* are distinct parasites.

---

[1] The signs (OO) and (P_4) indicate corpuscles containing respectively two rounded and four or more piriform parasites.

Observations on the blood.

In the case of *P. caballi* Horse III (refer to protocol and chart in the Appendix, p. 92) there was a relatively slight decrease in the number of red blood corpuscles (from about 6½ to about 5 millions) and a slight fall in the amount of haemoglobin. The leucocytes showed a decided decrease as the disease advanced, accompanied by a disappearance of the eosinophiles and an increase in the neutrophiles.

III. **Immunity test with regard to N. equi and P. caballi.**

As a final proof of the difference between the two parasites, we decided to test if an animal which had recovered from the symptoms of "biliary fever" produced by the one would prove immune to infection with the other parasite.

A horse inoculated with *P. caballi* on 22. VI. 1910 suffered from a mild infection, and to all outward appearances made a good recovery. On 2. VIII. 1910[1] this horse was inoculated with *N. equi* with the result that it died of "biliary fever" induced by this parasite 20 days later. Only *N. equi* could be found in this horse's blood, although it might have been expected that *P. caballi* would have reappeared. This was the only one of our six horses which had haemoglobinuria, and it is possible that the mixed infection which existed was responsible for this symptom. It is desirable to determine if haemoglobinuria occurs in a pure *N. equi* infection; it probably does, since the animals may show jaundice, but there may be a difference in respect to the frequency and severity of this symptom.

It would be of considerable interest to determine if trypanblue affects *P. caballi* in a similar manner to *P. canis* and *P. bovis*[2] as one of us has shown elsewhere. The dye may, moreover, be found to influence *N. equi* differently to *P. caballi*. We have been unable to investigate this problem owing to lack of means with which to continue the research[3].

We have pleasure in acknowledging the kind assistance given to us

---

[1] Forty-two days after it had been inoculated with *P. caballi*.

[2] *Vide* Nuttall and Hadwen, *Parasitology*, II. 156–191, 229–235, 236–266 and Nuttall, *Ibid.* II. 409–434 and III. 202–209.

[3] *Note whilst going through the press:* Dr Yakimoff, February, 1912, in a letter to one of us, states that Bielitzer has recently treated equine piroplasmosis successfully in Russia by means of trypanblue, following the suggestion of Nuttall and Hadwen. This dye has now been found of value in the treatment of canine, bovine and equine piroplasmosis.

by Sir John McFadyean and Dr E. J. Marzinowsky, who sent us the strains of parasites, *N. equi* and *P. caballi*, respectively, with which we worked. H. B. Fantham, D.Sc., and Miss A. Porter, D.Sc., and Mr B. G. Clark aided considerably in the earlier part of the investigation, and our thanks are also due to them for their assistance. We are furthermore indebted to Mr Joseph Barcroft, F.R.S., for the kind help he gave us in determining the haemoglobin content of the blood of our horses; he has supplied us with the Note on the method we employed which will be found described at the end of the Appendix.

In a future paper we shall give a general summary of our present knowledge regarding Nuttalliosis and Piroplasmosis in horses as observed in different parts of the world.

CONCLUSIONS.

The term "Biliary Fever" or "Piroplasmosis," hitherto supposed to apply to a specific disease affecting horses, in reality refers to two distinct diseases produced by distinct parasites. For convenience' sake, and in accordance with the terminology at present in vogue, these two diseases may be named after the parasites which produce them, *i.e.* Piroplasmosis (due to *Piroplasma* [or *Babesia*] *caballi* Nuttall, 1910) and Nuttalliosis (due to *Nuttallia equi* (Laveran, 1910), França, 1909).

REFERENCES.

BOWHILL, T. (10. I. 1905). Equine Piroplasmosis, or "Biliary Fever." *Journ. Hygiene*, v, 7–17, Pls. I–III.

FRANÇA, C. (1909). Sur la classification des Piroplasmes et description de deux formes de ces parasites. *Arch. do R. Inst. Bacteriol. Camara Pestana* (Lisbon), III, 11–18, Pl. II.

KOCH, R. (23. X. 1905). Vorläufige Mitteilungen über die Ergebnisse einer Forschungsreise nach Ostafrika. *Deutsche med. Wochenschr.*, XXXI, 1865, *et seq.*

LAVERAN, A. (1901). Contribution à l'étude de *Piroplasma equi*. *C. R. Soc. de. biol.* S. XII, 385–388; also *Rec. méd. vétérin.*, VIII, 380.

NUTTALL, G. H. F. (II. 1910). Note on the mode of multiplication of *Piroplasma bovis* as observed in the living parasite. *Parasitology*, II, 341–343, 1 Diagram.

NUTTALL, G. H. F. and GRAHAM-SMITH, G. S. (VI. 1908). The mode of multiplication of *Piroplasma bovis* and *P. pitheci* in the circulating blood compared with that of *P. canis*, with notes on other species of *Piroplasma*. *Parasitology*, I, 134–142, Pl. XI and Diagrams I–IV.

NUTTALL, G. H. F. and STRICKLAND, C. (7. XII. 1910). Die Parasiten der Pferdepiroplasmose resp. der "Biliary Fever." Vorläufige Mitteilung. *Centralbl. f. Bakteriol. u. Parasitenk.*, Originale. LVI, 524–525.

DESCRIPTION OF PLATE III.

Nuttallia equi. Illustrating chiefly its mode of multiplication in the circulating blood, as shown in Giemsa-stained blood-films. Drawings in water-colour, made by C. Strickland with the aid of a camera-lucida and selected from a large number of figures made from freshly-stained films.

Fig. 1. Small free parasite.

Fig. 2. Small parasite shortly after its entry into a corpuscle.

Figs. 3–8. Successive stages in the growth of parasites which may be either amoeboid (Figs. 5, 6) or assume more or less temporarily the piriform shape (Figs. 4, 7, 8).

Figs. 9–19 indicate successive stages in nuclear division. The chromatin mass, which has hitherto been rounded or oval, becomes elongated and bent in conformity with the external contour of the parasite (Figs. 9, 10); the terminal and middle portion of the chromatin band becomes swollen (Fig. 11), and the strands connecting the three growing masses become thinner (Fig. 12), whilst the central mass in turn sub-divides. In some cases (Figs. 13–15) the strand leading to one of the smaller masses of chromatin ruptures before the central mass shows signs of division; in other cases (Figs. 16, 17) the central mass may show signs of division at this stage. In the next stage (Figs. 18, 19) that which was the central mass is clearly divided, but a fine chromatin thread still connects the daughter nuclei.

Figs. 20–22. Nuclear division is here complete, there being four distinct masses of chromatin which are usually of about the same size; the blue-staining protoplasm is beginning to collect around each nucleus.

Fig. 23. A typical cross-form, with the chromatin masses situated at the ends of the cross which is formed of four pear-shaped elements joined at their tapering extremities.

Figs. 24, 25. The four young parasites have now separated within the corpuscle, no residual body being discernible.

Figs. 26–28. Groups of four parasites derived from cross-forms which have escaped from ruptured corpuscles. In Fig. 28 one of the four parasites has become separated from the group.

Fig. 29. Two free parasites which would presumably have entered a fresh corpuscle either singly or together.

Figs. 30–32. Stages of division leading up to the formation of three or possibly four parasites.

Figs. 33, 34. Groups of three small parasites inside (33) and outside (34) a corpuscle.

Fig. 35. Unusual appearance presented by two fairly large parasites, both of which are preparing to undergo division as evidenced by the structure of the chromatin. These parasites would have presumably given rise to six or eight parasites.

Figs. 36–40. Rounded, piriform or amoeboid parasites which show the chromatin drawn out and situated peripherally; 36 and 37 show a central vacuole.

Figs. 41, 42. Corpuscles containing two and three parasites respectively.

Figs. 43–45. Appearances suggesting that simple division of one cell into two may occasionally take place.

Figs. 46, 47. Single piriform parasites closely resembling *Piroplasma.*

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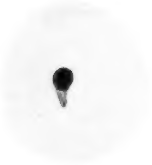

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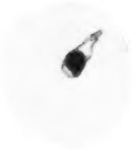

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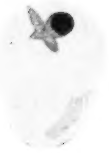

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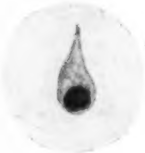

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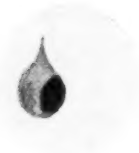

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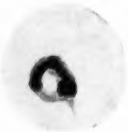

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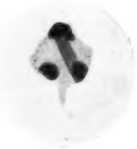

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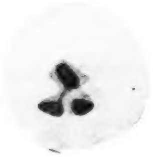

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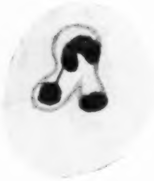

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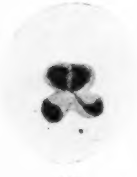

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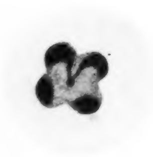

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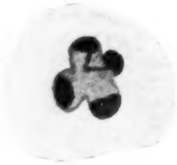

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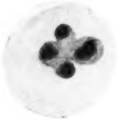

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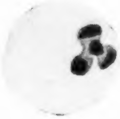

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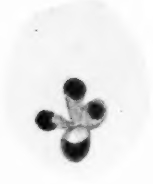

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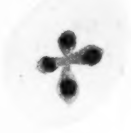

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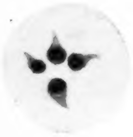

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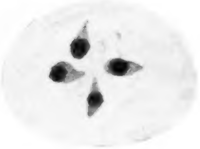

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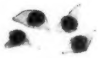
C. Strickland, del.

PLATE III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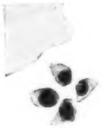
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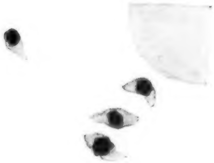
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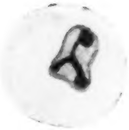
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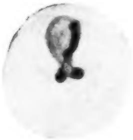
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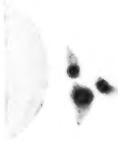
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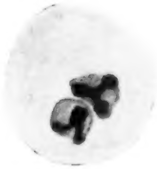
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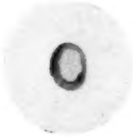
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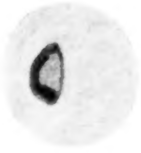
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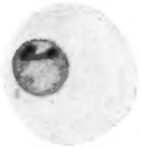
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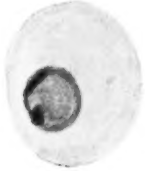
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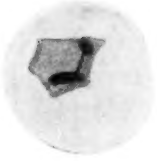
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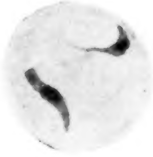
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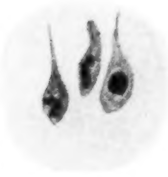
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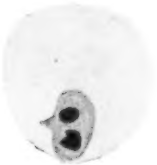
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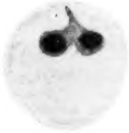
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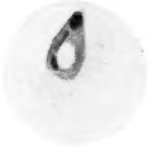
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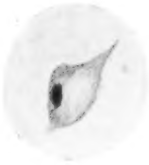
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APPENDIX.

CONTAINING THE PROTOCOLS AND TEMPERATURE CHARTS OF *N. EQUI* HORSES I—III AND *P. CABALLI* HORSES I—III.

Nuttallia equi Horse I.

The horse was inoculated subcutaneously with 5 c.c. of citrated blood at 10 a.m. on 27. XI. 1909; the horse died 7. XII. 1909 (day 11).

	Temp. °F.		
Day	M.	E.	
1	99·2	—	Inoculated.
2	100	—	
3	99·6	—	
4	99·6	—	
5	99·4	—	
6	100	—	
7	101·2	—	
8	102·6		Parasites appeared, very few, only 5 infected r.b.c. along 2 film-edges.

			°/₀ r.b.c. infected	°/₀						
Day	M.	E.		(S)	(M)	(L)	(D)	(2—4)	(F)	
8		103·4	0·57							
9	102·4		0·72	29	34	32	0·4	2	·	
		103·8	1·4	8	32	47	·	5	8	
10	105		4·8	2	50	36	2	3	1	
		105·8	5·4	3	31	62	·	4	4	8.30 p.m. horse weak on hind legs.
11	105		3·7	29	39	17	·	5	10	2 p.m. ditto, not feeding.
		104	3·9	55	11	4	0·4	3	26	6 p.m. prostrate; too weak to stand. Killed.

The signs denote corpuscles containing

(S) = A small intracorpuscular parasite
(M) = A medium-sized ditto
(L) = A large sized ,,
} rounded, piriform or amoeboid.
(D) = Dividing forms and cross-forms.
(2–4) = Two to four intracorpuscular parasites.
(F) = Free parasites.

From the following chart it will be seen that the parasites were first detected in the horse's blood on the 8th day after inoculation; their number rose so that about 5 °/₀ of the corpuscles were infected on the 10th day, whilst on the day following about 4 °/₀ were infected. With regard to the percentage of different types of parasites encountered, one striking fact emerges, namely, that, relatively speaking, the number of free and small parasites greatly increased toward death and the number of medium and large sized parasites decreased. The rise and fall in the percentage of infected corpuscles accompanied the rise and fall in the body temperature.

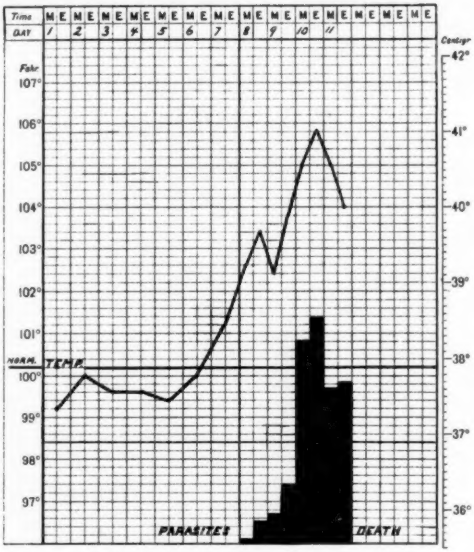

Chart of *Nuttallia equi* Horse I.

N. equi Horse II.

Horse inoculated subcutaneously with 5 c.c. of defibrinated jugular blood from Horse I at 5 p.m. 8. xII.1909. (Blood kept on ice 12 hours.)

	Temp. °F.		
Day	M.	E.	
1	—	—	Inoculated.
2	96·6	—	
3	100·6	—	
4	99·8	—	
5	99·6	—	
6	99·4	—	
7	102·6	102·6	Parasites appear, 0·04 % r.b.c. infected.
8	102·6	101·4	,, Morning 3 (O); Evening 5 (O).
9	101·0	101·8	,, ,, 19 (O), 1 (+), 1 (P).
10	104·2	103·8	,, ,, 0·9 % r.b.c. infected; Evening 0·7 %.
11	105·2	105·4	,, ,, 0·8 % ,, ,,
12	103·4	103·4	,, only 1 (O).
13	100·8	100·8	,, ,, 3 (O).
14	99·2	99·3	,, none found.
15	99·4	99·6	,, only 4 (O).
16	99·6	99·8	,, ,, 3 (O).
17	99·2	—	,, 14 (O), 1 (P).
18	98·8	—	,, 6 (O).
19	98·8	—	,, none found.
20	98·6	—	,, ,, ,,
21	99·4	—	,, 20 (O), 2 (P).
22–57	Normal		,, none found on 28 out of 35 days, temperature not taken on 7 days out of the 35.
58–148	,,		Not examined for parasites; temperature recorded on 61 out of the 90 days.

The signs (O), (P) and (+) indicate, respectively, single round, piriform (or oval) and cross-forms contained in corpuscles.

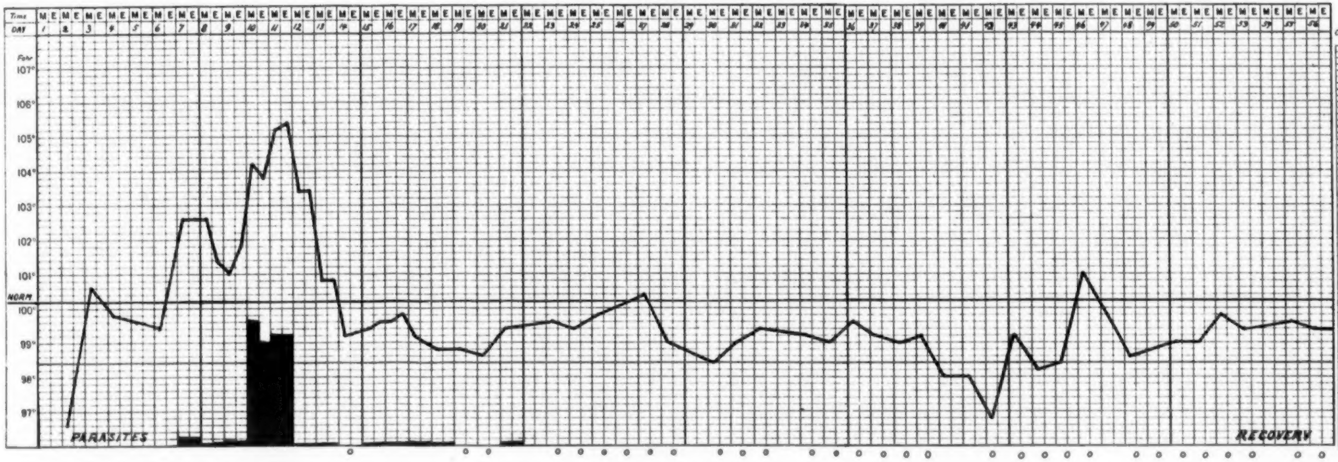

Chart of *Nuttallia equi* Horse II.

Reference to the foregoing chart shows that the parasites appeared with the onset of fever on the 7th day following the inoculation. The highest temperatures and highest percentages of infected corpuscles (only about 1 °/₀) were both attained on the 10th and 11th days, after which the temperature fell gradually to normal and the parasites dwindled in numbers. The small number of parasites precluded any enumeration of the relative percentages of the different types of parasites present in the blood. This animal made a complete recovery, and is alive and well to-day (27 February, 1912).

Nuttallia equi Horse III.

The Horse was inoculated subcutaneously with 20 c.c. of defibrinated blood taken from the jugular vein of Horse II at mid-day on 2. viii. 1910. Horse III had previously been infected with *Piroplasma caballi* on 22. vi. 1910, but had recovered (see Protocol of *P. caballi* Horse II). *N. equi* appeared in the horse's blood on the 10th day, and it died at 5.30 p.m. on the 20th day after inoculation (21. viii. 1910).

Day	Temp. °F. M.	Temp. °F. E.	°/₀ r.b.c. infected	% (S)	% (L)	% (D)	% (2—4)	% F		R.b.c. per per c.mm.	Haemo-globin	Leuco-cytes per c.mm.*
1	97·8	—							Inoculated.			
2	98·2	—										
3	98·2	—										
4	97·2	--										
5	98·2	—										
6	99·0	—										
7	98·2	—								8,888,000	0·227	8,640
8	98·2	—								8,872,000	0·230	12,900
9	97·4	—								8,968,000	0·222	7,120
10	100·4	—							Parasites appeared	9,155,000	0·240	7,800
10		102·0	0·2	34	65	1	·	·				
11	103·0		3·0	23	75	1	·	0·4		9,004,000	—	10,650
		104·2	2·0	43	50	4	2	·	Horse weak.			
12	104·2		—	—	—	—	—	—	Horse weak, jaundice; off his food.	7,240,000	0·230	9,540
		106·6	11·6	23	70	3	4	1				
13	105·0		10·3	47	45	0·6	2	5	Very weak; haemoglobinuria; blood appears watery, coagulation retarded.	5,390,000	—	—
		104·6	6·8	50	43	2	3	2				
14	103·0		8·4	56	38	0·2	4	1	Rather better; feeding better, haemoglobinuria.	5,365,000	0·141	12,000
		101·7	10·6	62	33	2	2	1				
15	100·6		5·2	31	65	1	3	0·4	Ditto; urine smoky.	4,812,000	0·104	7,520
		102·4	7·4	28	66	0·6	5	0·6				
16	102·4		9·2	15	80	0·6	3	0·2	Very weak, especially hind legs, which are stretched apart.	3,840,000	0·115	9,700
		100·8	6·8	35	57	1	4	1				
17	103·0		8·0	19	75	1	3	0·2	Seems better, feeding; swelling on jaw (abscess).	3,848,000	0·104	13,500
		103·0	12·8	28	67	2	3	·				
18	103·0		8·6	25	69	1	4	0·3	Weak; jaundice persists.	3,230,000	0·100	22,600
		102·2	13·2	20	73	2	5	·				
19	103·2		6·6	23	74	1	2	·	Very weak, lying down.	3,120,000	0·066	33,600
		101·0	4·8	20	75	1	4	·				
20	98·4		8·4	29	53	2	11	6	Helpless, urine smoky; found dead at 5.30 p.m.		Mean of two readings, being the O capacity of 1 c.c. of blood in c.c.	

* Average number of counts = 5.

The signs denote corpuscles containing

(S) = small and medium sized piriform or oval parasites.
(L) = large rounded parasites.
(D) = dividing and cross-forms.
(2–4) = two or more parasites.
F = free forms.

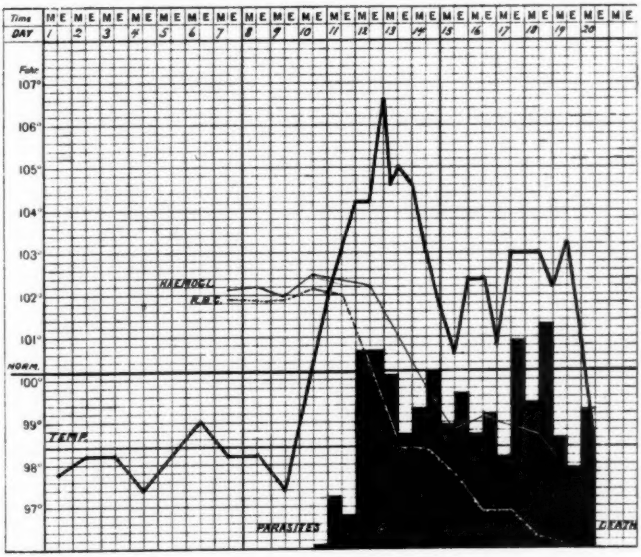

Chart of *Nuttallia equi* Horse III.

Changes in the blood.

	Differential leucocyte count, %					
Day	Lymphocytes	Large mono's	Transit.	Polym's	Eosin's	Nucleated r.b.c.
8	27	32	2	26	13	0
14	20	29	8	43	0	0
17	26·5	34	14	22	1	3
19	22	34	7·3	34·4	0	3·3

Piroplasma caballi Horse I.

The Horse was inoculated subcutaneously at 3 p.m., 4. vi. 1910, with 15 c.c. of citrated blood sent to us by Dr E. J. Marzinowsky, from Moscow. The horse died on the 22. vi. 1910 (day 19).

Day	Temp. °F.	
1	—	Inoculated.
2	100·4	
3	97·8	Abscess forming at seat of inoculation.
4	99·1	
5	97·4	Abscess lanced.
6	96·8	
7	97·4	
8	96·2	
9	97·0	
10	97·2	
11	96·8	
12	97·4	
13	97·8	
14	99·8	
15	101·0	Parasites appeared, only 2 (PP) found after long search.
16	99·6	
17	100·0	Parasites present, only 3 (PP) found.
18	101·4 (a.m.)	,, ,, few, 66 °/o (O), 34 °/o (PP).
	102·4 (p.m.)	
19	—	Horse found dead but still warm at 9 a.m. *Died of pulmonary haemorrhage.*

There were too few parasites present in this horse's blood to render a satisfactory blood-count possible during life. Of the parasites found on the 18th day, 66 °/o were single round parasites (O) and 34 °/o double piriforms (PP). It was possible, however, in stained films prepared from the internal organs after death, to make an enumeration of the different types of parasites as follows:

	(O)	(D)	(PP)	Free
Liver	84	1	8	7
Spleen	73	2	10	15
Lung	77	0	3	20

Piroplasma caballi Horse II.

The horse was inoculated subcutaneously at 5.30 p.m., 22. VI. 1910, with 20 c.c. of defibrinated blood taken from the jugular vein of *P. caballi* Horse I very soon after death. Recovered quickly.

Day	Temp. °F. a.m.	Temp. °F. p.m.	
1	97·2	—	Inoculated.
2	96·8	—	
3	99·2	—	
4	98·2	—	
5	97·2	—	
6	97·6	—	
7	100·8	—	
8	102·4	103·4	Parasites found, only 1 (O), 3 (PP). Horse off his feed.
9	100·8	101·8	No parasites found.
10	96·8	97·4	,, ,,
11	100·4	102·4	,, ,,
12	100·8	101·4	,, ,,
13	98·8	98·6	,, ,,
14	100·2	98·4	,, ,,
15	100·2	100·0	,, ,,
16	99·8	99·6	,, ,,
17	99·8	99·8	,, ,,
18	97·6	100·6	,, ,,
19	Normal		,, ,, The horse recovered and showed no subsequent rise of temperature. Forty-two days after it was inoculated with *P. caballi* blood it was inoculated with *N. equi* blood; the further history of the horse is given under *N. equi* Horse III.

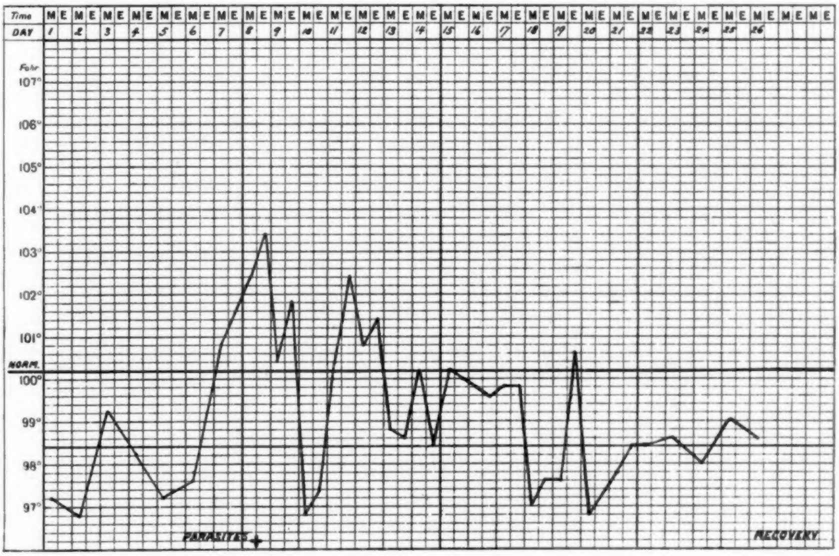

Chart of *Piroplasma caballi* Horse II.

In this horse the temperature began to rise on the 7th day and parasites were found on the 8th day, although only four infected corpuscles were encountered in a blood-film. No parasites were detected after the 10th day, and the temperature soon returned to normal.

Piroplasma caballi Horse III.

The Horse was inoculated subcutaneously at 12.30 p.m., 18. VII. 1910, with 20 c.c. of defibrinated blood taken from *P. caballi* Horse II. The horse died at 2.30 p.m., on 5. VIII. 1910 (day 19).

Day	Temp. °F.		No. of infected r.b.c. per 30,000	%					
	a.m.	p.m.		(P)	(O)	(D)	(PP)	F	
1	99·8	—							Inoculated.
2	98·8	—							
3	99·2	—							
4	100·2	—							
5	99·0	—							
6	99·0	—							
7	99·0	—							
8	98·6	—							
9	100·8								
		103·0							
10	103·4								Parasites appeared, only 1 (PP) found in film.
		103·2							,, ,, ,, 1 (PP) ,, ,,
11	102·2								,, ,, ,, 1 (P), 1 (PP) found in film. Feeding less.
		101·8							
12	102·2								,, ,, ,, 1 (O), 1 (PP) ,, ,,
		102·0							
13	100·0	—	6						
14	99·0	—	3						Horse feeding very little.
15	104·6	—	12						,, ,, ,,
16	105·4	—	6	6	71	2	21	0	,, ,, ,,
		105·6	12	6	47	5	42	0	
17	105·4		4	13	67	4	15	1	Horse weak on hind legs, drinks much.
		105·0	60	5	64	4	27	0	
18	103·6		85	10	68	4	18	0	Horse cannot stand; icterus; spleen large and tender.
		102·2	80	4	57	7	31	0	
19	102·4		150 = 0·5 %	2	51	7	39	1	Ditto, some normal urine passed.
		—	Treated at 1.30 p.m. by subcutaneous injection of 150 c.c. of 1·5 % Trypanblue solution. Horse died at 2 p.m.						

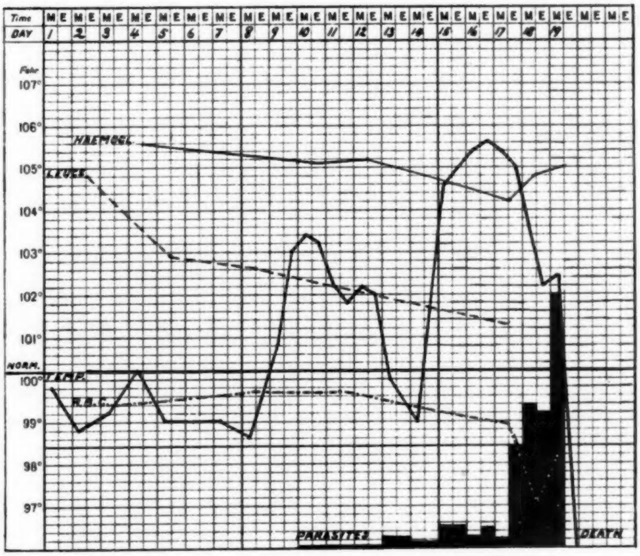

Chart of *Piroplasma caballi* Horse III.

Changes in the blood.

				Differential count of leucocytes		
Day	R.b.c. per c.mm.	Haemoglobin	Leucocytes per c.mm.	Eosin's	Neutroph's	Mono's and lympho's
1	—	—	—	30	53	17
2	—	—	8,800			
3	6,400,000	—	—			
4	—	0·207	—			
5	6,570,000	—	6,850			
8	6,700,000	—	6,600			
10	—	0·189	—			
11	6,685,000	—	—			
12	—	0·192	—			
17	5,872,000	0·155	5,300	0	83	17
18	4,140,000	0·178	—			
19	4,960,000	0·185	—			
		Mean of two readings in each case, being O capacity of 1 c.c. of blood in c.c.	Mean of ca., ten readings in each case.			

From the foregoing record we see that the body temperature rose on the 9th day, and that the parasites appeared on the 10th day after inoculation. Following upon the initial rise of temperature there was a fall and then a rise, the temperature attaining 105·6° F. on the 16th day. The red blood corpuscles were not materially decreased in numbers except toward the end, and the haemoglobin content was not appreciably altered. There was a fall in the number of leucocytes. The parasites multiplied rapidly only during the last 36 hours, but they never infected more than 0·5 $^0/_0$ of the corpuscles.

Note upon the chemical method of haemoglobin determination used in this investigation.

The oxygen capacity of the blood was investigated by direct measurement of the oxygen which it contained when shaken with air. The method employed was that form of the differential method described by Barcroft and Roberts (*Journ. of Physiology*, xxxix. p. 435, 1910) and based upon the observation of Haldane, that ferricyanide of potassium liberated, from an alkaline solution of haemoglobin, an amount of oxygen equal to the respiratory oxygen contained combined with the pigment.

The method is briefly as follows:

Into each of the bottles is placed 0·2 c.c. of dilute ammonia solution (made by adding 1 c.c. of NH_3, SG ·880 to 250 c.c. of water) and about 0·1 c.c. of blood, accurately measured from a pipette. The present experiments were performed with 0·082 c.c. The fluids are gently shaken so that the blood becomes laked and thoroughly oxidised. The bottles may then be placed on the apparatus, but before this is done a drop of potassium ferricyanide is placed in the pouch projecting from the side of one of the bottles. The ferricyanide is introduced with a curved pipette, and care must be taken to see that no mixture takes place between the solution of ferricyanide and haemoglobin. The apparatus is now hung on the edge of a vessel full of water so that the bottles are completely immersed. After ten minutes the taps, which hitherto have allowed both the manometer and the bottles to be in communication with the external air, are closed so that the two former are in communication with one another but not with the air outside. The fluid surfaces in the manometer are read to ascertain if any zero error exists. They are now at the same level. The ferricyanide and the haemoglobin are mixed and the apparatus shaken for a minute and then replaced in the bath. Probably three such shakings suffice to liberate all the oxygen. The observed difference of pressure is noted. The estimation is then repeated with the blood in the other bottle.

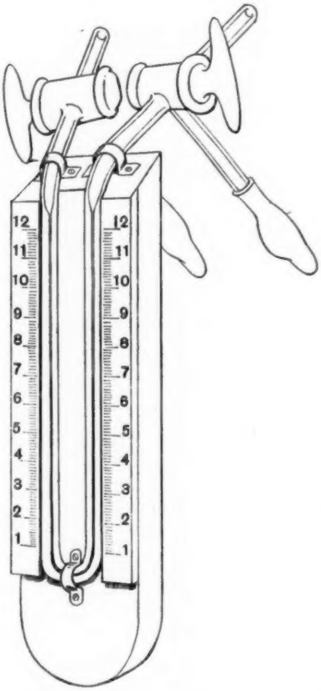

Fig. 1. Showing the apparatus used in the method here described.

To the right of the figure is seen one and part of another of the small flasks in which the mixture of blood, ammonia, and ferricyanide is made. The manometer and the flasks and the air are seen to be connected by three-way passages governed by three-way cocks. The dark line in the manometer represents the oil. (Figure reproduced from the *Journ. of Physiol.*, by kind permission of Professor Langley.)

It is necessary to know how much oxygen corresponds to a difference of pressure of 1 mm. on the scale. For this purpose a known volume of oxygen is liberated in the apparatus (about 0·2 c.c.). An experiment similar to that described, but with different fluids, is performed. The blood is replaced by hydrogen peroxide, the ammonia by water and the ferricyanide by potassium permanganate (approximately 1 c.c. = 0·003 g. permanganate). The hydrogen peroxide must first be carefully standardised by titration with standard potassium permanganate in the presence of sulphuric acid with which reagent it gives off its oxygen as follows:

$$K_2Mn_2O_8 + 5H_2O_2 - 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 5H_2O + 5O_2.$$

In the apparatus itself it is better to dispense with the sulphuric acid. The relation of 316 grams of permanganate to 1110·00 c.c. of oxygen remains true.